Week 8

July 25, 2016

What We Did Today:

Ran Gibson Assembly with ADARs and Neg Control (Using 0.8 of the original concentration)

Made Liquid Cultures from Gibson Apobec Plates

Will Digest with EcoR1 and xHo tomorrow to run on gel

Gibson Assembly Mixtures

ADAR 1-1x

0.98 ul pRETRo-ON
1.52 ul dCas9 Insert
2.12 ul ADAR 1-1x Insert
8 ul Mastermix
3.38 ul Water

ADAR 1-2x

0.98 ul pRETRo-ON
1.52 ul dCas9 Insert
3.56 ul ADAR 1-2x Insert
8 ul Mastermix
1.94 ul Water

ADAR 1-3x

0.98 ul pRETRo-ON
1.52 ul dCas9 Insert
1.62 ul ADAR 1-3x Insert
8 ul Mastermix
3.88 ul Water

ADAR 2-1x

0.98 ul pRETRo-ON
1.52 ul dCas9 Insert
3.65 ul ADAR 2-1x Insert
8 ul Mastermix
1.85 ul Water

ADAR 2-2x

0.98 ul pRETRo-ON
1.52 ul dCas9 Insert
3.48 ul ADAR 2-2x Insert
8 ul Mastermix
2.02 ul Water

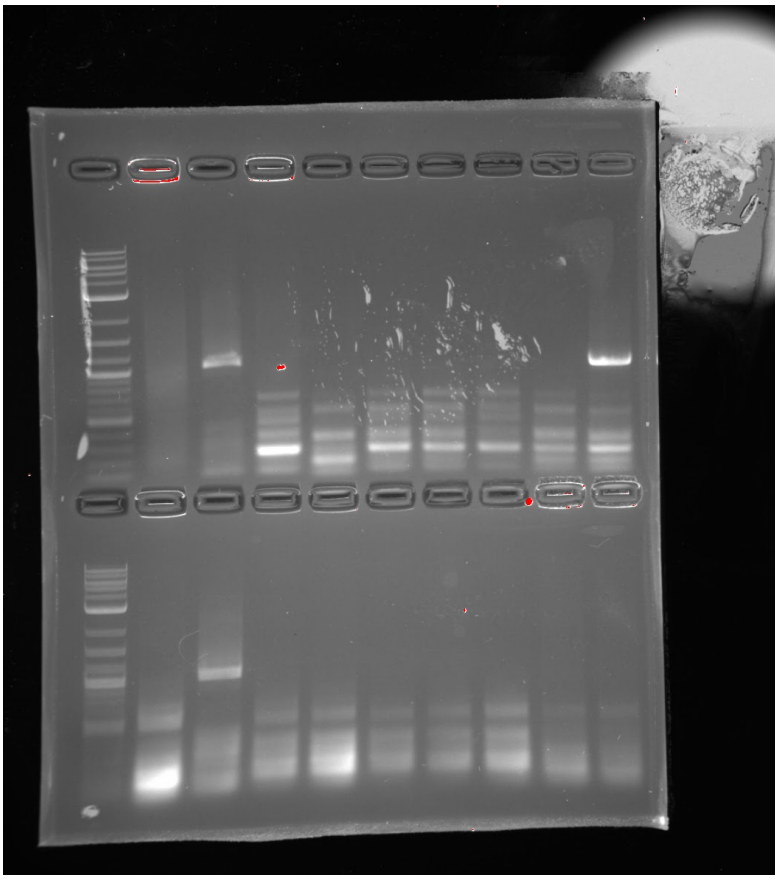
ADAR 2-3x
- 0.98 ul pRETRo-ON
- 1.52 ul dCas9 Insert
- 3.35 ul ADAR 2-3x Insert
- 8 ul Mastermix
- 2.15 ul Water

Negative Control
- 0.98 ul pRETRO-ON
- 8 ul Mastermix
- 7.02 ul Water

APOBEC 1x
- 0.98 ul pRETRO-ON
- 1.52 ul dCas9 Insert
- 3.34 ul APOBEC 1x Insert
- 8 ul Mastermix
- 2.16 ul Water

Colony PCR again
One successful wild type clone with a clear insert

Picked it and grew in liquid culture



Pick colonies from 10uL ligation for liquid cultures

Transformed eGFP samples
Set up 12 well plate of HEK293T cells for transfection

- Digest
  dCas9-ADAR ligations (7 samples from 7/22)
- Gel
  Run for >1.5 hours

July 26, 2016

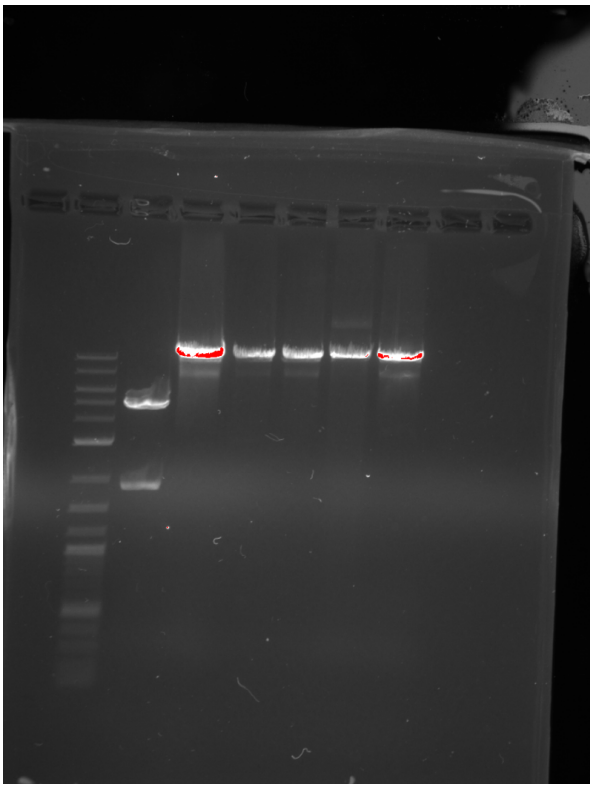
What We Did Today:

- Counted Gibson Plates
- Made liquid cultures of Gibson plates
- Mini-prepped Apobec 2x and 3x Gibson
- Digested Apobec 2x and 3x with EcoR1 and xHo1
- Ran Digests on gel
- Interviews

Gibson Plate Counts

- Adar 2-1x
  1. 2
  2. 0
  3. 3
- Adar 2-2x
  1. 1
  2. 4
  3. 2
- Adar 1-2x
  1. 10
  2. 7
  3. 18
- Adar 2-3x
  1. 11
  2. 9
  3. 2
- Neg Control
  1. 0
  2. 0
  3. 0
- Apobec 1x
  1. 5 (none single)
  2. 8
  3. 1
- Adar 1-1x
  1. 11
  2. 7
  3. 3
- Adar 1-3x
  1. 6
  2. 8
  3. 12

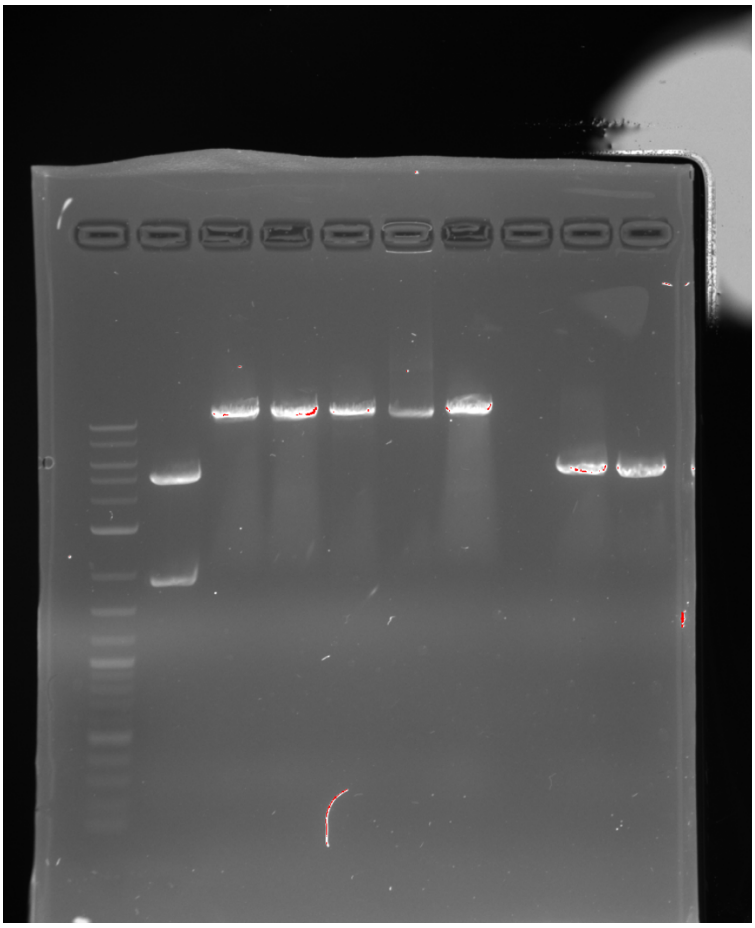
Gel 1



Gel 1: Apobec 2x
Lane 1 – Empty
Lane 2 – Ladder
Lane 3 – Neg. Control
Lane 4 – Apobec 2x #1
Lane 5 – Apobec 2x #2
Lane 6 – Apobec 2x #3
Lane 7 – Apobec 2x #4
Lane 8 – Apobec 2x #5

Analysis: The Apobecs ran correctly!! They are around where we would expect the 12kb marker to be. We realized that the EcoR1 site was cut out during the Gibson Assembly.

Gel 2

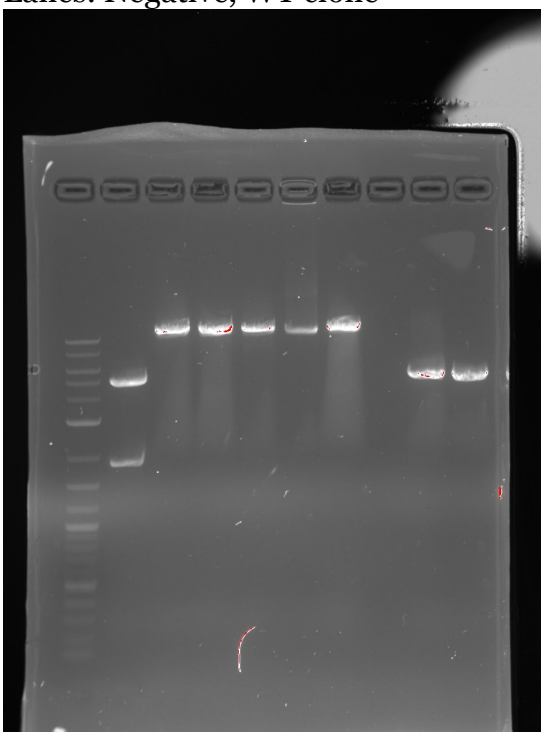


Gel 2: Apobec 3x
Lane 1 – Ladder
Lane 2 – Neg. Control
Lane 3 – Apobec 3x #1
Lane 4 – Apobec 3x #2
Lane 5 – Apobec 3x #3
Lane 6 – Apobec 3x #4
Lane 7 – Apobec 3x #5
Lane 8 – Empty
Lane 9 – Frederick Neg
Lane 10 – Frederick test

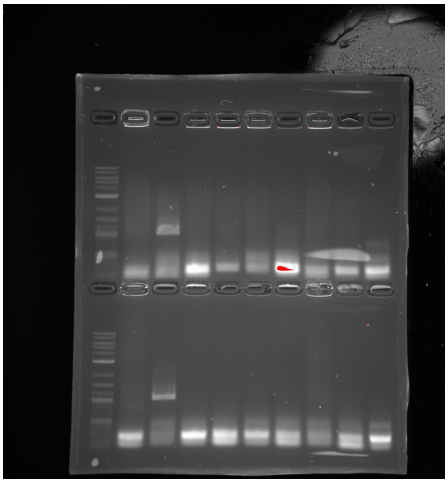
Analysis: The Apobecs ran correctly!! They are around where we would expect the 12kb marker to be. We realized that the EcoR1 site was cut out during the Gibson Assembly.

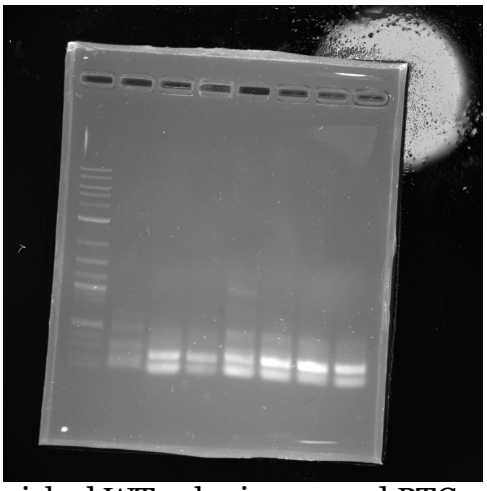
Wild type colony digested with XhoI and BamHI

AND THE INSERT DISAPPEARED
Lanes: Negative, WT clone



screened 21 more PTC colonies to try to find a clone
didn't find one





picked WT colonies 1-7 and PTC 7 just in case something got mixed up

Test digest:
14uL water
2uL DNA
2uL buffer
1uL each enzyme

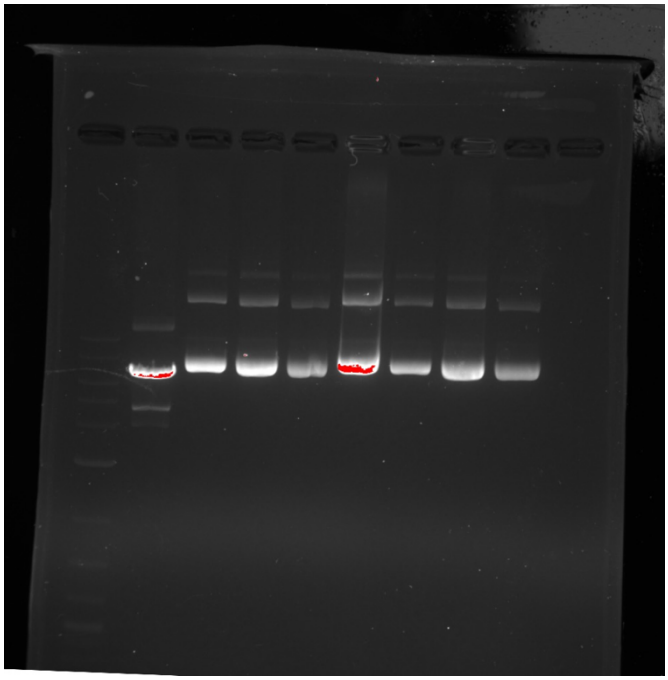
Made liquid cultures of eGFP colonies in 2x AMP

- Glycerol stocks
- Miniprep
  - dCas9-ADAR/APOBEC ligations (8 samples)
    - Concentration: ~130-330 ng/µL
- Digest
  - Enzymes: ECoRI, XbaI
    - 0.5 µL each enzyme
    - 4 µL DNA (dCas9-ADAR/APOBEC)
    - 1 µL CutSmart buffer
  - (10 µL total)
- Gel

July 27, 2016

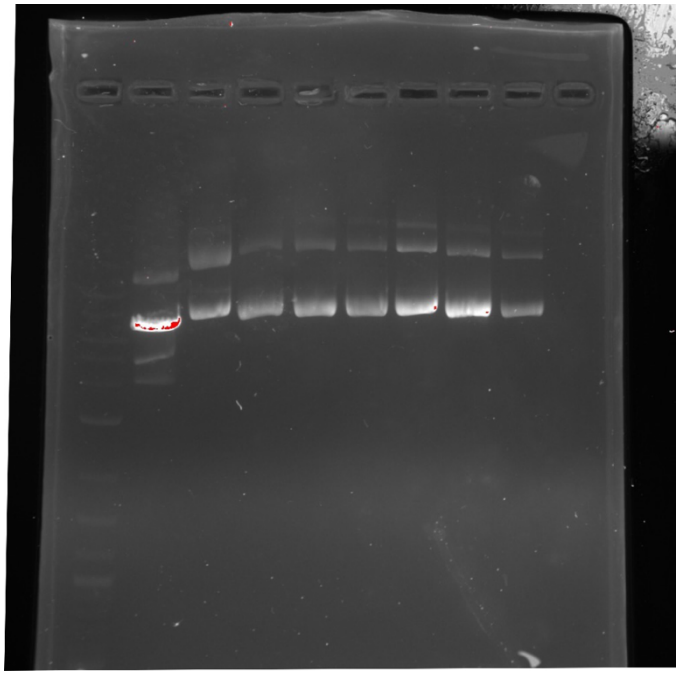
What We Did Today:

- Mini-prepped Apobec 1x and Adar 1-1x, 1-2x, 1-3x, 2-1x, 2-2x, 2-3x Gibson
- Digested Apobec 1x and Adar 1-1x, 1-2x, 1-3x, 2-1x, 2-2x, 2-3x xHo1
- Ran Digests on gel
- Made 2x Amp Plates



Gel 1
Lane 1 – Ladder
Lane 2 – Neg. Control
Lane 3 – Adar 1-1x #1
Lane 4 – Adar 1-1x #2
Lane 5 – Adar 1-1x #3
Lane 6 – Adar 1-2x #1
Lane 7 – Adar 1-2x #2
Lane 8 – Adar 1-2x #3
Lane 9 – Apobec 1x #1

Analysis
Did not run well



Gel 2
Lane 1 – Ladder
Lane 2 – Neg. Control
Lane 3 – Adar 1-3x #1
Lane 4 – Adar 1-3x #2
Lane 5 – Adar 1-3x #3
Lane 6 – Adar 2-1x #1
Lane 7 – Adar 2-1x #2
Lane 8 – Adar 2-1x #3
Lane 9 – Apobec 1x #2

Analysis
No clones

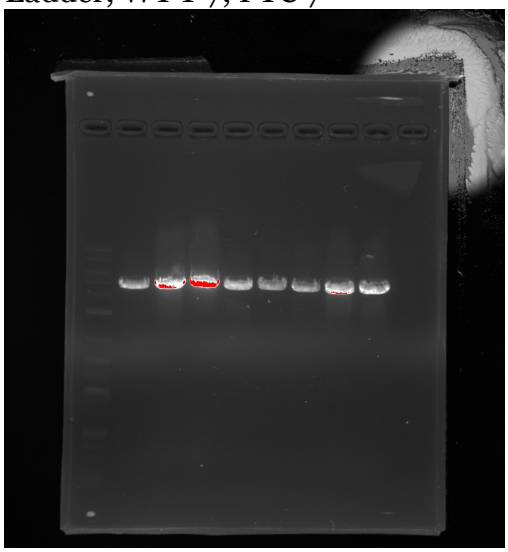
Gel 3
Lane 1 – Ladder
Lane 2 – Neg. Control
Lane 3 – Adar 2-2x #1
Lane 4 – Adar 2-2x #2
Lane 5 – Adar 2-2x #3
Lane 6 – Adar 2-3x #1
Lane 7 – Adar 2-3x #2
Lane 8 – Adar 2-3x #3
Lane 9 – Apobec 1x #3

Analysis
No clones

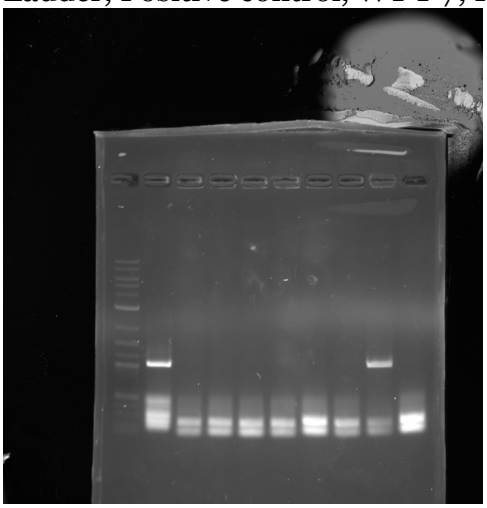
Colony PCR the 8 liquid cultures, 10uL of liquid culture brought up to 25uL with 15uL water, boiled then typical procedure

digested 2uL of each (after miniprep) with XhoI and BamHI

Gel 1:
Digest:
Ladder, WT 1-7, PTC 7



Gel 2:
Colony PCR
Ladder, Positive control, WT 1-7, PTC 7



The globin came back, then was gone again

July 28th, 2016

What We Did Today:

PCR dcas

PCR Mixture

1.) Primers were diluted 1:10 with water (9ul water, 1ul Primer)

2.) Components were added to PCR tubes

1.) 22ul Water

2.) 1ul Template (ADAR 1, ADAR 2, and APOBEC with all extensions)

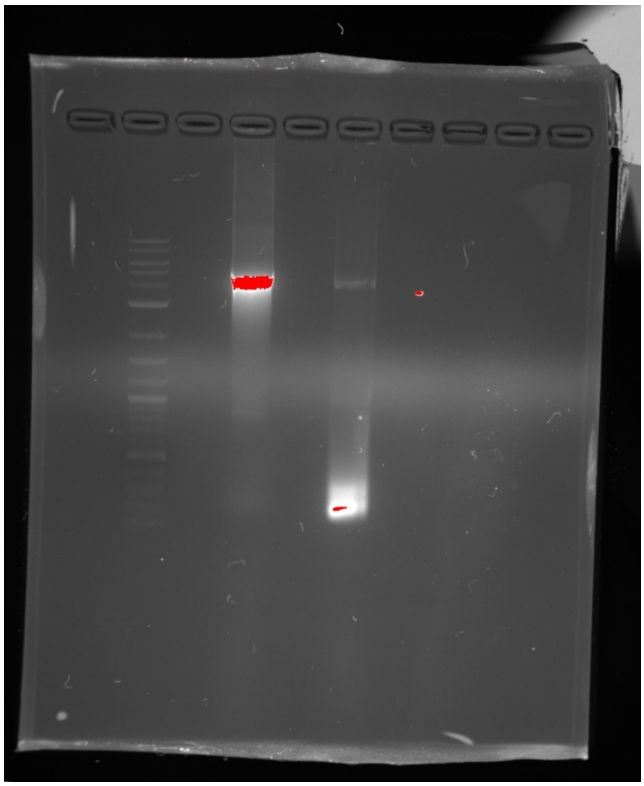
3.) 1ul Primer 1 (3' XTen)

4.) 1ul Primer 2 (5' dCas)

5.) 25 ul MasterMix

3.) Tubes were run in the PCR Machine under the iGEM Colony Protocol for 50ul tubes

4.) 5ul of each sample was run on a gel



Gel – dCas9 Insert Testing
Lane 1 – Empty
Lane 2 – Ladder
Lane 3 – Empty
Lane 4 – Old dCas9 Insert
Lane 5 – Empty
Lane 6 – New dCas9 Insert

Analysis
The old dCas9 insert ran correctly however the new one did not. We can only see the primer dimers for this sample.

- Fixing cells

eGFP trial 1

- 6 slides
- Positive, negative, ATG, ACG

- Imaging
  - 2 fields/cover slip

- Green/red fluorescence channel each
- 5X objective

48 images total
Sequenced globin trials, editing enzymes
Examined transfected cells
Fixed and mounted transfected cells

Created standard curve for Interlab using FITC and made standardization samples for Interlab
Measured samples using plate reader at gateway

July 29, 2016

Gibson Mixtures

Adar 1-1x

1.23 ul pRETRO-ON
1.90 ul dCas Insert
2.65 ul Adar Insert
10 ul Mastermix
4.22 ul Water

Adar 1-2x

1.23 pRETRO-ON
1.90 dCas9 Insert
4.44 ul Adar Insert
10 ul Mastermix
2.43 ul Water

Adar 1-3x

1.23 ul pRETRO-ON
1.90 dCas9 Insert
2.03 ul Adar Insert
10 ul Mastermix
4.84 u. Water

Adar 2-1x

1.23 pRETRO-ON
1.90 dCas9 Insert
4.57 ul Adar Insert
10 ul Mastermix
2.3 ul Water

Adar 2-2x

1.23 ul pRETRO-ON
1.90 dCas9 Insert
4.35 ul Adar Insert
10 ul Mastermix
2.52 ul Water

Adar 2-3x

1.23 ul pRETRO-ON
1.90 dCas9 Insert
4.18 ul Adar Insert
10 ul Mastermix
2.69 ul Water

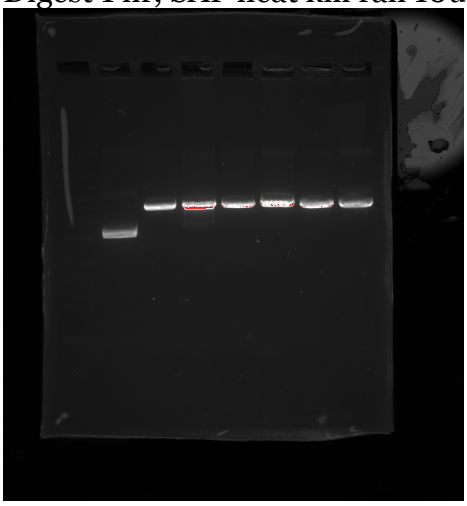
Apobec 1x

1.23 ul pRETRO-ON
1.90 dCas9 Insert
4.18 ul Apobec Insert
10 ul Mastermix
2.69 ul Water

Neg. Control

1.23 pRETRO-ON
10 ul Mastermix
8.77 ul Water

Test digest of globin vector with BglII, Xho1, HindIII, BamHI, EcoRI, PstI to test for efficiency of the cutting of the enzyme
Digest 1 hr, SAP heat kill ran 10uL on a gel



ligate 5 mins 1uL
Gel:
L, neg, Bam, Bgl, Eco, Hind, Pst, Xho

Mini prepped additional pgRNA
Maintenance of HEK293T cells